RESEARCH PLAN PROPOSAL

STUDY OF THE EFFECT OF NANOPARTICLES USED IN FOODSTUFFS IN SWISS ALBINO MICE AND PREVENTIVE ACTION OF *ALOE VERA*

For registration to the degree of

Doctor of Philosophy

IN THE FACULTY OF LIFE SCIENCE

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Submitted by

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INTRODUCTION

Nanotoxicology is a branch of bio - nanoscience that deals with the study and application of the toxicity of nanomaterials (Buzea *et al.,* 2007). It is a sub - specialty of particle toxicology. It addresses the toxicology of nanoparticles, which appear to find toxic effects that are unusual and not seen with larger particles.

Nanomaterials are materials of a single - sized unit (in at least one dimension) between 1 and 1000 nanometers (10^{-9} meter) but it is usually 1 - 100 nm (Buzea *et al.,* 2007). The definition adopted by the International Organization for Standardization (ISO), 2010 is "Material with any external dimension in the nanoscale or having internal structure in the nanoscale (size range from 1 nm to 100 nm)". Nanomaterials have unique properties because of quantum size and large surface area to volume ratio. The extremely small size of nanomaterials also means that they much more readily gain entry into the human body than larger sized particles. Nanoparticles can be inhaled, swallowed, or absorbed through skin, and deliberately or accidentally inject during medical procedures.

Holsapple *et al.* (2005) suggested that nanomaterials were able to cross biological membranes and access cells, tissues and organs that larger - sized particles normally cannot. Nanomaterials could gain access to the blood stream via inhalation (Oberdorster *et al.,* 2005a) or ingestion (Hoet *et al.,* (2004). Then, once in the blood stream, nanomaterials could transported around the body and taken up by organs and tissues, including the brain, heart, liver, kidneys, spleen, bone marrow, and nervous system (Oberdorster *et al.,* 2005b). Unlike larger particles, nanomaterials taken up by the cell mitochondria (Li *et al.,* 2003) and the cell nucleus (Porter *et al.,* 2007; Geiser *et al.,* 2005).

Size is a key factor in determining the potential toxicity of a particle. However, it is not the only important factor. Other properties of nanomaterials that influenced toxicity were chemical composition, shape, surface structure, surface charge, aggregation and solubility, (Nel *et al.,* 2006) and the presence or absence of functional groups of other chemicals (Magrez *et al.,* 2006). Buzea *et al.* (2007) suggested that adverse effects of nanoparticles on human health depend on individual factors such as genetics and existing disease, as well as exposure, and nanoparticle chemistry, size, shape, agglomeration state, and electromagnetic properties. The key to understanding the toxicity of nanoparticles is their minute size (smaller than cells and cellular organelles) allows them to penetrate basic biological structures, disrupting their normal function. Nanostructures could activate the immune system by inducing inflammation, immune responses, allergy, or even affect to the immune cells in a deleterious or beneficial w ay (immune - suppression in autoimmune diseases, improve immune responses in vaccines).

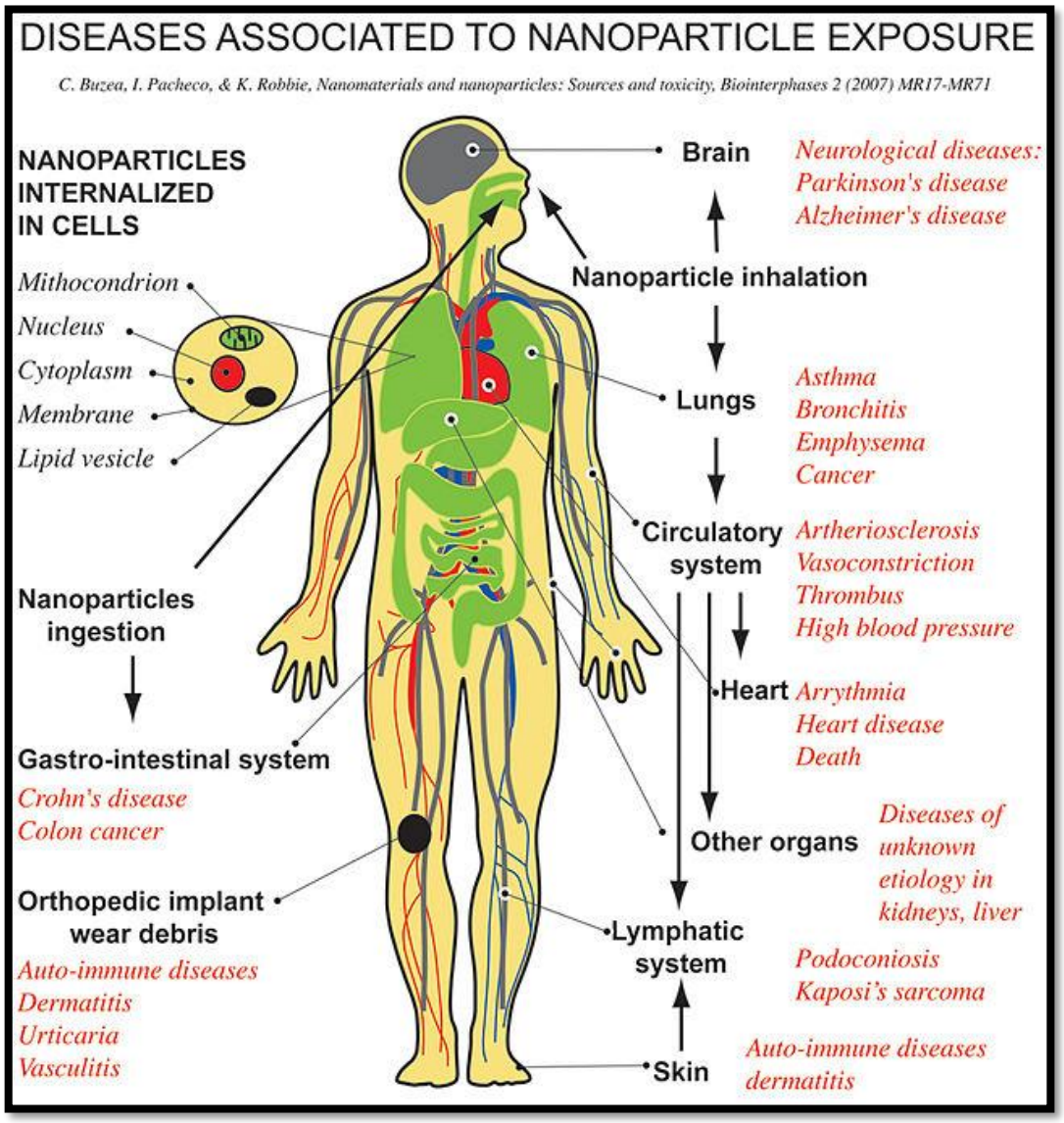


Figure 1: Pathways of exposure to nanoparticles and associated diseases as suggested by epidemiological, *in vivo,* and *in vitro* studies (Buzea *et al.*, 2007).

The Royal Society identified the potential for nanoparticles to penetrate the skin, and recommended that the use of Nanoparticles in cosmetics. These materials increasingly being used for commercial purposes such as fillers, pacifiers, catalysts, semiconductors, cosmetics, microelectronics, and *drug carriers* (Nel *et al.,* 2006). A range of nanoparticles investigated for biomedical applications including tissue engineering, drug delivery, and biosensor (Kerativitayanan *et al.,* 2015). Nanoparticles have also attached to textile fibers in order to create smart and functional clothing (Heim *et al.,* 2015). Zinc oxide particles have found to have superior UV blocking properties compared to its bulk substitute. This is one of the reasons why it was used in the preparation of sunscreen lotions, and it is completely photo stable (Mitchnick *et al.* 1999).

Titanium dioxide is also known as titanium (IV) oxide or titania. It is the naturally occurring oxide of titanium. Its chemical formula is TiO_2. It has a wide range of applications,

from paint to sunscreen to *food coloring*. The most important application areas are paints and varnishes as well as paper and plastics, which account for about 80% of the world's titanium dioxide consumption. Other pigment applications such as printing inks, fibers, rubber, cosmetic products, and *foodstuffs* account for another 8%. The rest used in other applications, such as production of technical pure titanium, glass, and glass ceramics, electrical ceramics, catalysts, electric conductors and chemical intermediates (Market Study, 2013). It is also present in *red - colored candy*. It is also an effective opacifier in powder form, where it has employed as a pigment to provide whiteness and opacity to products such as paints, coatings, plastics, papers, inks, *foods*, medicines, and toothpastes. Phillips *et al.* (1997) suggested that titanium dioxide increase skimmed milk's whiteness by increasing skimmed milk's sensory acceptance score.

Nanoparticle research is currently an area of intense scientific interest due to a wide variety of potential applications in biomedical, optical and electronic fields (Taylor *et al.*, 2013a; 2013b; 2012); Hewakuruppu *et al.*, 2013). It is necessary to develop specialized approaches to testing and monitoring their effects on human health and on the environment. How nanoparticles behave inside the body is still a major question that needs to be resolved.

REVIEW OF LITERATURE

As the use of nanomaterials increases worldwide, concerns for worker and user safety are mounting. To address such concerns, various scientists conducted various studies.

Dunkin' Donuts in the United States is dropping titanium dioxide from its powdered sugar donuts after public pressure (Isidore, 2015; Filloon, 2015; Horovitz, 2015). However, Andrew Maynard (2015) disregarded extreme danger from the use of titanium dioxide in food. He suggested that the titanium dioxide used by Dunkin' Brands and many other food producers was not a new material or not a nanomaterial. Nanoparticles were typically smaller than 100 nanometers in diameter but most of the particles in food grade titanium dioxide were much larger.

Kerativitayanan *et al.* (2015) and Lauterwasser (2007) reported unwanted effects such as increased rate of absorption of nanomaterials while nanomaterials and nanotechnologies are expecting to yield numerous health and health care advances, such as more targeted methods of delivering drugs, new cancer therapies, and methods of early detection of diseases. The greater specific surface area (surface area per unit weight) might lead to increased rate of absorption through the skin, lungs, or digestive tract and might caused unwanted effects to the lungs as well as other organs.

Dixon (2013) and Mnyusiwalla *et al.* (2003) suggested that nanoparticles presented possible dangers, both medically and environmentally. Most of those were due to the high surface to volume ratio, which could make the particles very reactive or catalytic (Ying, 2001). Berglund *et al.* (2011) showed that the rare disease yellow nail syndrome might caused by titanium which implanted either for medical reasons or through eating various *foods containing titanium dioxide*.

Zook *et al.* (2011) dispersed gold, silver, cerium oxide, and positively – charged polystyrene nanoparticles in cell culture media, by using the timing between mixing steps to control agglomerate size in identical media. They demonstrated the importance of controlling agglomeration by shown large agglomerates of silver nanoparticles caused significantly less hemolytic toxicity than small agglomerates.

Yazdi *et al.* (2010) and University of California (2009) found that titanium dioxide nanoparticles caused inflammatory response and genetic damage in mice. Hanley *et al.* (2009) looked at the effects of ZnO nanoparticles on human immune cells, and found varying levels of susceptibility to cytotoxicity.

In a study, Poland *et al.* (2008) introduced carbon nanotubes into the abdominal cavity of mice. Their results demonstrated that long thin carbon nanotubes showed the same effects as

long thin asbestos fibers. They raised concerns that exposure to carbon nanotubes might lead to pleural abnormalities such as mesothelioma (cancer of the lining of the lungs caused by exposure to asbestos).

Satariano (2008) found that Diesel nanoparticles had damage the cardiovascular system in a mouse model. Murray (2008) reported potential health risks due to many sunscreens that used titanium dioxide nanoparticle and zinc oxide nanoparticle.

Buzea *et al.* (2007) showed that 'Humans have always been exposed to tiny particles via dust storms, volcanic ash, and other natural processes, and our body systems are well adapted to protect us from these potentially harmful intruders. The reticulo - endothelial system actively neutralizes and eliminates foreign matter in the body, including viruses and non - biological particles. Within the nearly limitless diversity of these materials, some happen to be toxic to biological systems; others are relatively benign; and others confer health benefits'.

In March 2004, Eva Oberdorster found extensive brain damage to fish exposed to fullerenes for a period of just 48 hours at a relatively moderate dose of 0.5 parts per million (commensurate with levels of other kinds of pollution found in bays). The fish also exhibited changed gene markers in their livers; indicating that their entire physiology was affected. In a concurrent test, the fullerenes killed water fleas (Oberdorster *et al.*, 2005b). In this work in July 2004, questions were raised of potential cytotoxicity of nanoparticles which were made of C_{60}, had now been shown by several sources to be likely caused by the tetrahydrofuran (THF) used in preparing the 30 nm - 100 nm particles of C_{60} used in the research. Isakovic *et al.* (2006) reviewed this phenomenon, gave results, which showed that removal of THF from the C_{60} particles resulted in a loss of toxicity. Sayes *et al.* (2007) also showed that particles prepared as in Oberdorster study caused no detectable inflammatory response when instilled intra - tracheally in rats after observation for 3 months. They suggested that even the particles prepared by Oberdorster do not exhibit markers of toxicity in mammalian models.

Kashiwada (2006) investigated the effects of nanoparticles on soft - bodied organisms. His study allowed him to explore the distribution of water - suspended fluorescent nanoparticles (solid latex solution) throughout the eggs and adult bodies of a species of fish, known as the see - through medaka (*Oryzias latipes*). For his study, Kashiwada noted that nanoparticles had taken up into the blood stream and deposited throughout the body. In the medaka eggs, there was a high accumulation of nanoparticles in the yolk; bioavailability was dependent on specific sizes of the particles. Adult samples of medaka had accumulated nanoparticles in the gills, intestine, brain, testis, liver, and blood stream when exposed to a10 mg/L nanoparticle solution. One major result from that study was that salinity might have a large influence on the bioavailability. His

results suggested that nanoparticles were capable of penetrating the blood - brain barrier and they eventually reach the brain.

Nel *et al.* (2006) suggested that nanomaterials were engineered structures with at least one dimension of 100 nanometers or less. Materials in this range might approach the length scale at which some specific physical or chemical interactions with their environment could occur. As a result, their properties differ substantially from those bulk materials of the same composition, allowed them to perform exceptional feats of conductivity, reactivity, and optical sensitivity. Possible undesirable results of these capabilities showed harmful interactions with biological systems and the environment, with the potential to generate toxicity.

Maynard (2006) reported that certain nanoparticles might move easily into sensitive lung tissues after inhalation, and caused damage that could lead to chronic breathing problems. Benson *et al.* (2005) demonstrated that zinc nanoparticles were not absorbed into the blood stream *in vivo*.

Oberdorster *et al.* (2005a) developed a screening strategy for the hazard identification of engineered nanomaterials. They suggested that physicochemical properties might be important in understanding the toxic effects of test materials include particle size and size distribution, agglomeration state, shape, crystal structure, chemical composition, surface area, surface chemistry, surface charge, and porosity. Tier 1 *in vivo* assays had proposed for pulmonary, oral, skin and injection exposures, and Tier 2 evaluations proposed for pulmonary exposures. Nanomaterials had proved toxic to human tissues and cell cultures, resulted in increased oxidative stress, inflammatory cytokine production, and cell death.

Morones *et al.* (2005) studied the effects of silver nanoparticles in the range of 1 - 100 nm on Gram - negative bacteria using high angle annular dark field (HAADF) scanning transmission electron microscopy (STEM). Their results indicated that the bactericidal properties of the nanoparticles are size dependent, since the only nanoparticles that present a direct interaction with the bacteria preferentially have a diameter of ~ 1 - 10 nm.

Some studies demonstrated the potential for nanomaterials to cause DNA mutation (Geiser *et al.*, 2005) and to induce major structural damage to mitochondria (Hoet *et al.*, 2004; Li *et al.*, 2003; Savic *et al.*, 2003).

Ballou *et al.* (2004) were tested quantum dots having four different surface coatings for used in *in vivo* imaging. They monitored localization by fluorescence imaging of living animals, by necropsy, frozen tissue sections for optical microscopy, and electron microscopy, on scales ranging from centimeters to nanometers, used only quantum dots for detection. Circulated half-lives found to be less than 12 min for amphiphilic poly (acrylic acid), short-chain (750 Da) methoxy-PEG or long-chain (3400 Da) carboxy-PEG quantum dots, but approximately 70 min

for long-chain (5000 Da) methoxy-PEG quantum dots. Surface coatings also determined the *in vivo* localization of the quantum dots. Long-term experiments demonstrated that these quantum dots remain fluorescent after at least four months *in vivo*.

Bazile *et al.* (1992) were prepared fully biodegradable poly lactic acid (PLA) nanoparticles (90–250 nm) coated with human serum albumin (HSA) by high-pressure emulsification and solvent evaporation. They used the protein as surfactant. They developed a new analytical tool based on Mie's law and size exclusion chromatography. After evaporation of the solvent, the protein saturated the surface of the nanoparticles, and masked the PLA core. According to that technique, no HSA has encapsulated in the polymer matrix. A radio labelled [^{14}C]-PLA$_{50}$ synthesized to follow the fate of that new drug carrier after *i.v.* administration to rats. The time necessary to clear the albumin-coated nanoparticles from the plasma was significantly longer than for the uncoated ones but not extended enough to target cells other than mononuclear phagocytes. As deduced from whole-body autoradiography and quantitative distribution experiments, the ^{14}C-labelled polymer rapidly captured by liver, bone marrow, lymph nodes, spleen and peritoneal macrophages. Nanoparticle degradation was addressed followed ^{14}C excretion. The elimination of the ^{14}C was quick on the first day (30% of the administered dose) but then slowed down. In fact, if the metabolism of the PLA proceeds to lactic acid that rapidly converted into CO_2 via the Krebs cycle (the lungs fulfilled 80% of the total excretion), anabolism from the lactic acid may also have taken place leading to long-lasting radioactive remnants, by incorporation of ^{14}C into endogenous compounds.

The affinity of nanoparticles for hematopoietic organs could be valuable for the targeting of certain stimulating factors to those tissues, but this affinity was also be taken into account in the toxicological evaluation of those carriers, especially when they are loaded with anti-mitotic compounds such as doxorubicin. Gibaud *et al.* (1996) studied the capture, the localization, and the retention in the bone marrow and in the spleen of biodegradable poly (isohexyl cyanoacrylate) nanoparticles as well as of non-biodegradable polystyrene nanoparticles. The histological localization of these nanoparticles has completed by cytological localization with a method used in cytochemistry for the evaluation of intracellular accumulation of various substances, such as iron deposits in bone marrow sideroblasts. They indicated that, in the bone marrow, after a quick passage through the endothelium, nanoparticles dispersed throughout in the tissue and captured by all types of phagocytized cells. In the spleen, nanoparticles have mainly localized in large angular capturing cells in the marginal zone of the lymphoid follicles.

Gibaud *et al.* (1994) were compared *in vivo* myelo-suppressive effects of free and polyalkylcyanoacrylate-bound doxorubicin in a mouse model. After intravenous administration of 11 mg/kg body weight of doxorubicin either free or bound to polyisobutyl (doxo-PIBCA) or

polyisohexylcyanoacrylate (doxo-PIHCA) nanoparticles, they studied the total and differential counts of blood, bone marrow and spleen cells; the number of granulocyte progenitors (CFU-GM) determined by culture. Doxorubicin concentrations measured with an HPLC method in the bone marrow and the spleen. Doxo-PIHCA nanoparticles showed the highest and longest myelo-suppressive effects that correlated well with a high concentration of the drug in the bone marrow and the spleen. They found that PIHCA nanoparticles induced the release of colony stimulating factors, which might account for the observed increase of toxic effects of doxorubicin on bone marrow progenitors. They also indicated that a more precise evaluation of the myelo-suppressive effects of targeted formulations of anticancer drugs is needed, which may be attained by studies on bone marrow progenitors.

The human recombinant granulocyte colony-stimulating factor (rhG-CSF) largely used in the treatment of neutropenia occurring during chemotherapy. After injection, this glycoprotein distributed through the whole body. Thus, Gibaud *et al.* (1998) obtained high and durable bone marrow concentrations, targeting with polyalkylcyanoacrylate nanoparticles. They were investigated two methods of preparation:

1. Anionic polymerization
2. Precipitation of the preformed polymer

By anionic polymerization, it was possible to associate more than 66% of rhG-CSF with nanoparticles (polyisobutyl- or polyisohexylcyanoacrylate nanoparticles) when the glycoprotein added at the end of the polymerization process. It has shown that the rhG-CSF mainly adsorbed on the surface of the nanoparticles and most of the colony stimulating activity conserved. Using precipitation of performed polyisohexylcyanoacrylate, 90% of rhG-CSF was associated with nanoparticles, the protein mainly adsorbed onto the nanoparticle surface. In this case, they observed a decrease of the colony stimulating activity. Whatever the method used, the *in vitro* release of rhG-CSF from the polyisohexylcyanoacrylate nanoparticles, was progressive during 8 h in seric conditions. Nevertheless, used mice as an animal model, it has been shown that its association with polyisohexylcyanoacrylate nanoparticles did not increase the short-term effects of intravenously injected rhG-CSF.

Au *et al.* (2009) has been demonstrated the therapeutic potential of transplantation of embryonic stem cells (ESCs) in animal model of myocardial infarction. They have used labeling of super paramagnetic iron oxide (SPIO) nanoparticles and cardiac magnetic resonance imaging (MRI) to track the migration of transplanted cells *in vivo* allowed cell fate determination. They demonstrated that ESCs labeled with SPIO compared to their unlabeled counterparts had similar cardiogenic capacity, and SPIO-labeling did not affect calcium-handling property of ESC-

derived cardiomyocytes. Transplantation of SPIO-labeled ESCs via direct intra-myocardial injection to infarct myocardium resulted in significant improvement in heart function.

Krejci and his colleagues in 2008 suggested that labeling of mammalian cells with super paramagnetic iron oxide (SPIO) nanoparticles enabled to monitor their fate *in vivo* using magnetic resonance imaging (MRI). They were investigated the effects of Endorem and Resovist SPIO nanoparticles on the growth and differentiation of mouse embryonic stem (ES) cells *in vitro*. Their observations have shown that SPIO nanoparticles have no effect on the self-renewal of ES cells. They also studied the effect of SPIO on the formation of embryoid bodies and neural differentiation of ES cell in monolayer culture. The cavitation of embryoid bodies has partially inhibited and neural differentiation was supported regardless the type of SPIO nanoparticles used.

Ciofani *et al.* (2010) obtained highly biocompatible and highly concentrated dispersions of boron nitride nano-tubes and tested on human neuroblastoma cells. A systematic investigation of the cytotoxicity of those nano-vectors with several complementary qualitative and quantitative assays allowed a strong interference with the MTT metabolic assay. Their results confirmed the high complexity of those new nanomaterials, and the needing of extensive investigations on their exciting potential applications in the biomedical field.

New materials of emerging technological importance are single-walled carbon nano-tubes (SWCNTs). Because SWCNTs will use in commercial products in huge amounts, their effects on human health and the environment have addressed in several studies. Inhalation studies *in vivo* and submerse applications *in vitro* have been described with diverging results. Knirsch *et al.* (2006) reported why some indicate a strong cytotoxicity and some do not. Data from human alveolar epithelial A549 cells incubated with carbon nano-tubes fake a strong cytotoxic effect within the MTT assay after 24 h that reached roughly 50%, whereas the same treatment with SWCNTs, but detection with WST-1, reveals no cytotoxicity. Lactate dehydrogenase (LDH), FACS-assisted mitochondrial membrane potential determination, and Annexin-V/PI staining also revealed no cytotoxicity. SWCNTs appeared to interact with some tetrazolium salts such as MTT but not with others (such as WST-1, INT, and XTT). This interference does not seem to affect the enzymatic reaction but lies rather in the insoluble nature of MTT-formazan. Their findings strongly suggested verifying cytotoxicity data with at least two or more independent test systems for that new class of materials (nanomaterials). They intensely recommended standardizing nano-toxicological assays with regard to the material used. MTT-formazan crystals formed in the MTT reaction were lumped with nano-tubes and offered a potential mechanism to guide bioremediation and clearance for SWCNTs from “contaminated”

tissue. SWCNTs are good supporting materials for tissue growth, as attachment of focal adhesions and connections to the cytoskeleton suggested.

Lee *et al.* (2010) were achieved patterned growth of boron nitride nano-tubes (BNNTs) by catalytic chemical vapor deposition (CCVD) at 1200 °C using MgO, Ni, or Fe as the catalysts, and an Al_2O_3 diffusion barrier as under layer. The as-grown BNNTs had high crystallinity and vertically aligned. Near band-edge absorption, ~6.0eV detected, without significant sub-band absorption centers. Electronic transport measurement confirmed that those BNNTs are perfect insulators, applicable for future deep-UV photo electronic devices and high-power electronics.

Gold nanoparticles (GNPs) offer a great promise in biomedicine. Reeves *et al.* (2010) were evaluated the bioaccumulation and toxic effects of different doses (40, 200, and 400 µg/kg/day) of 12.5 nm GNPs upon intra-peritoneal administration in mice every day for 8 days. The gold levels in blood did not increase with the dose administered, whereas in all the organs examined there was a proportional increase on gold, indicated efficient tissue uptake. Although brain was the organ containing the lowest quantity of injected GNPs, their data suggested that GNPs are able to cross the blood–brain barrier and accumulated in the neural tissue. They did not observed any evidence of toxicity in any of the diverse studies performed, including survival, behavior, animal weight, organ morphology, blood biochemistry and tissue histology. The results indicated that tissue accumulation pattern of GNPs depend on the doses administered and the accumulation of the particles did not produced sub-acute physiological damage.

Titanium dioxide (TiO_2) is widely used. Morishige *et al.* (2010) described that its inhalation can induce inflammatory diseases accompanied by interleukin-1β (IL-1β) production. The particle characteristics of TiO_2 are important factors in its biological effects. It is urgently necessary to investigate the relationship between the particle characteristics and biological responses for the development of safe forms of TiO_2. They systematically compared the production of IL-1β in response to various forms of TiO_2 by macrophage-like human THP-1 cells using various sizes (nano to micro), crystal structures (anatase or rutile), and shapes (spherical or spicular) of TiO_2. The production of IL-1β depended dramatically on the characteristics of the TiO_2. Smaller anatase and larger rutile particles provoked higher IL-1β production. In addition, IL-1β production depended on active cathepsin B and reactive oxygen species production independent of the characteristics of TiO_2. Their results provided basic information for the creation of safe and effective novel forms of TiO_2.

Du *et al.* (2012) reported that extensive use of silver nanoparticles for antimicrobial applications; cellular mechanisms underlying microbial response to silver nanoparticles remains to be further elucidated at the systems level. They reported systems-level response of

Escherichia coli to silver nanoparticles using transcriptome based biochemical and phenotype assays. They provided the evidence that anaerobic respiration induced upon exposure to silver nanoparticles. Then, they showed that anaerobic respiration-related regulators and enzymes play an important role in *E. coli* resistance to silver nanoparticles. In particular, their results suggested that *arcA* is essential for resistance against silver NPs and the deletion of *fnr, fdnH* and *narH* significantly increased the resistance. They envisaged that their study offers novel insights into modes of antimicrobial action of silver nanoparticles, and cellular mechanisms contributing to the development of microbial resistance to silver nanoparticles.

Pulskamp *et al.* (2007) focused on carbon nano-tubes (CNTs), which represent one of the most widely investigated carbon nanoparticles. Their data indicated that CNTs are able to cross the cell membrane of rat macrophages (NR8383) and might have an influence on cell physiology and function. NR8383 and human A549 lung cells were incubated with commercial single-walled (NT-1) and multi-walled (NT-2, NT-3) CNTs. They did not observe any acute toxicity on cell viability (WST-1, PI-staining) upon incubation with all CNT products. None of the CNTs induced the inflammatory mediators (NO, TNF-alpha and IL-8). A rising tendency of TNF-alpha released from LPS-primed cells due to CNT treatment observed. They detected a dose and time-dependent increase of intracellular reactive oxygen species and a decrease of the mitochondrial membrane potential with the commercial CNTs in both cell types after particle treatment whereas incubation with the purified CNTs (acid-treated single-walled CNT) had no effect. That leads them to the conclusion that metal traces associated with the commercial nano-tubes were responsible for the biological effects.

Yoshida *et al.* (2012) demonstrated that nanomaterials have utilized in various fields. In particular, amorphous nano-silica particles are increasingly being used in a range of applications, including cosmetics, food technology, and medical diagnostics. There is concern that the unique characteristics of nanomaterials might induce undesirable effects. The roles played by the physical characteristics of nanomaterials in cellular responses have not yet elucidated precisely. By using nano-silica particles (nSPs) with a diameter of 70 nm whose surface was either unmodified (nSP70) or modified with amine (nSP70-N) or carboxyl groups (nSP70-C), they examined the relationship between the surface properties of nSPs and cellular responses such as cytotoxicity, reactive oxygen species (ROS) generation, and DNA damage. To compare the cytotoxicity of nSP70, nSP70-N, or nSP70-C, they examined *in vitro* cell viability after nSP treatment. Although the susceptibility of each cell line to the nSPs was different, nSP70-C and nSP70-N showed lower cytotoxicity than nSP70 in all cell lines. The generation of ROS and induction of DNA damage in nSP70-C- and nSP70-N-treated cells were lower than those in nSP70-treated cells were. Their results suggested that the surface properties of nSP70 play an

important role in determining its safety, and surface modification of nSP70 with amine or carboxyl groups may be useful for the development of safer nSPs.

Kang *et al.* (2009) investigated the signaling pathways underlying nano-TiO_2-induced apoptosis in cultured human lymphocytes. Nano-TiO_2 increased the proportion of sub-G1 cells, activated caspase-9 and caspase-3, and induced caspase-3-mediated PARP cleavage. Nano-TiO_2 also induced loss of mitochondrial membrane potential, which suggested that nano-TiO_2 induced apoptosis via a mitochondrial pathway. A time-sequence analysis of the induction of apoptosis by nano-TiO_2 revealed that nano-TiO_2 triggered apoptosis through caspase-8/Bid activation. They also observed that inhibition of caspase-8 by z-IETDfmk suppressed the caspase-8/Bid activation, caspase-3-mediated PARP cleavage, and apoptosis. Nano-TiO_2 activated two MAPKs, p38 and JNK. In addition, the selective p38 inhibitor SB203580 and selective JNK inhibitor SP600125 suppressed nano-TiO_2-induced apoptosis and caspase-8 activation to moderate and significant extents, respectively. Knockdown of protein levels of JNK1 and p38 using an RNA interference technique also suppressed caspase-8 activation. Their results suggested that nano-TiO_2-induced apoptosis was mediated by the p38/JNK pathway and the caspase-8-dependent Bid pathway in human lymphocytes.

The increasing amount of nanotechnological products, found in our environment and those applicable in engineering, material sciences and medicine has stimulated a growing interest in examining their long-term impact on genetic and epigenetic processes. Gong *et al.* (2010) examined the epigenomic response to nm- SiO_2 particles in human HaCaT cells and methyltransferases (DNMTs) and DNA-binding domain proteins (MBDs) induced by nano- SiO_2 particles. Nm- SiO_2 treatment induced global hypoacetylation implying a global epigenomic response. The levels of DNMT1, DNMT3a and methyl-CpG binding protein 2 (MBD2) were also decreased in a dose dependent manner at mRNA and protein level. Epigenetic changes might have long-term effects on gene expression programming long after the initial signal has removed, and if those changes remain undetected, it could lead to long-term untoward effects in biological systems. Their studies suggested that nanoparticles could cause more subtle epigenetic changes that merit thorough examination of environmental nanoparticles and novel candidate nanomaterials for medical applications.

Though nanomaterials is not confirmed as a health risk to workers who produce those, NIOSH recommended that exposure use; precautions and personal protective equipment to protect workers until the risks of nanomaterial manufacture are better understood (Topmiller *et al.*, 2013).

OBJECTIVES

- To study the effects (if any) of the test substance that is titanium dioxide which is prevalently used in making foodstuffs

- To find its effects at different dose levels

- To find haematological; biochemical and histo - pathological changes in liver and intestine due to it, if any

- To find the role of *Aloe vera* in nullifying its effects

METHODOLOGY

❖ Experimental Animal:-

Swiss Albino Mice (approximately 6 weeks old) from the Animal House of The IIS University (CPCSEA Registration No.: 1689/PO/9/13/CPCSEA).

❖ Drug:-

Sub - Chronic Administration of Nano Titanium Dioxide (TiO_2) of different concentrations.

❖ Dose Administration:-

Dose will given by an oral administration to the experimental animal.

❖ Dose:-

1. 6 mg titanium dioxide with 1 ml distilled water
2. 12 mg titanium dioxide with 1 ml distilled water
3. 24 mg titanium dioxide with 1 ml distilled water
4. *Aloe vera* juice for preventive action (0.01 ml twice in a day)

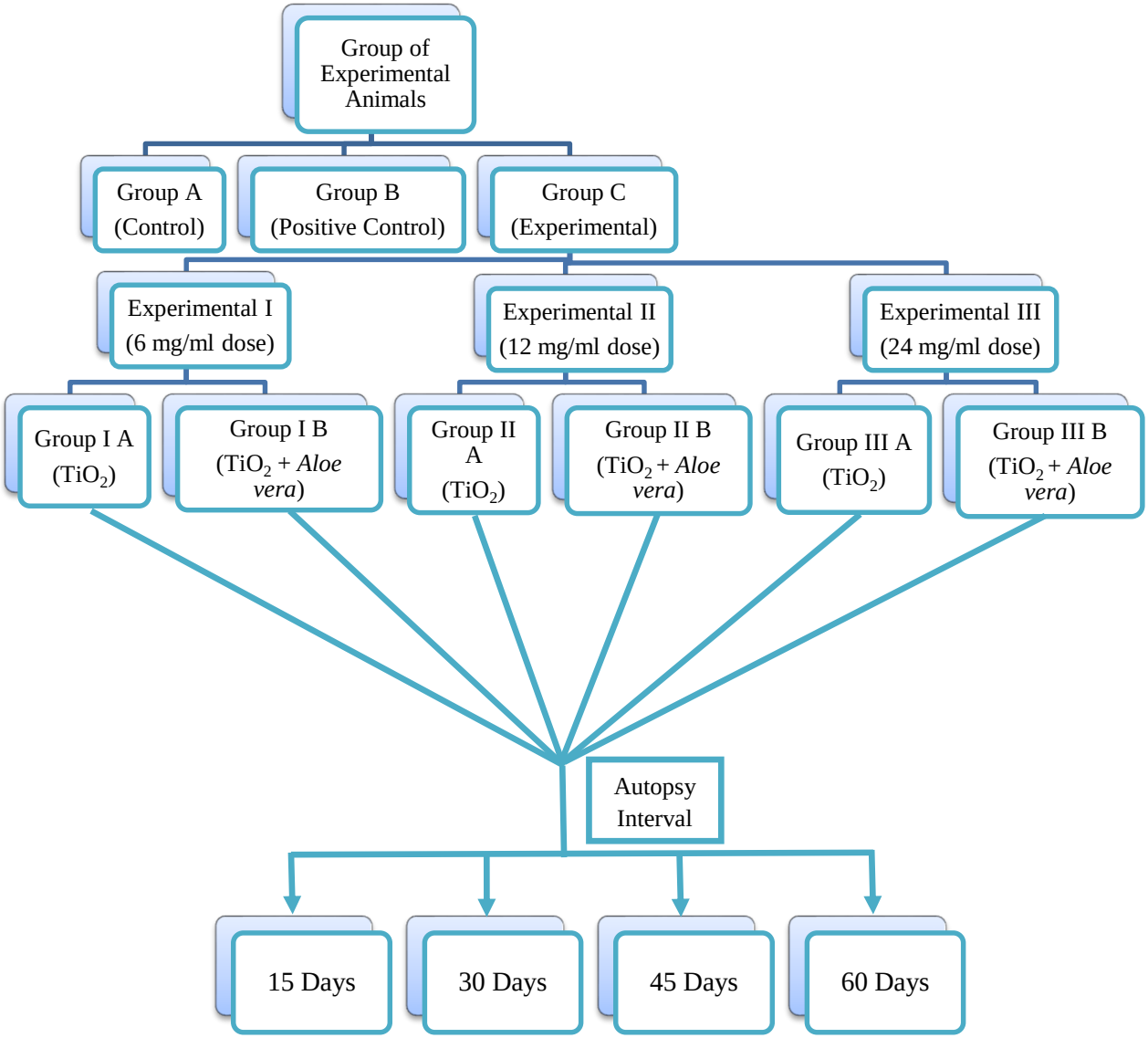
❖ Autopsy Interval:-

15 Days, 30 Days, 45 Days, and 60 Days.

❖ There will be five groups for experiments:-

1. **Group I:** Normal (Control) Group
2. **Group II:** Positive Control Group
3. **Group III A:** Experimental group treated with 6 mg TiO_2
4. **Group III B:** Experimental group treated with 6 mg TiO_2 + *Aloe vera*
5. **Group IV A:** Experimental group treated with 12 mg TiO_2
6. **Group IV B:** Experimental group treated with 12 mg TiO_2 + *Aloe vera*
7. **Group V A:** Experimental group treated with 24 mg TiO_2
8. **Group V B:** Experimental group treated with 24 mg TiO_2 + *Aloe vera*

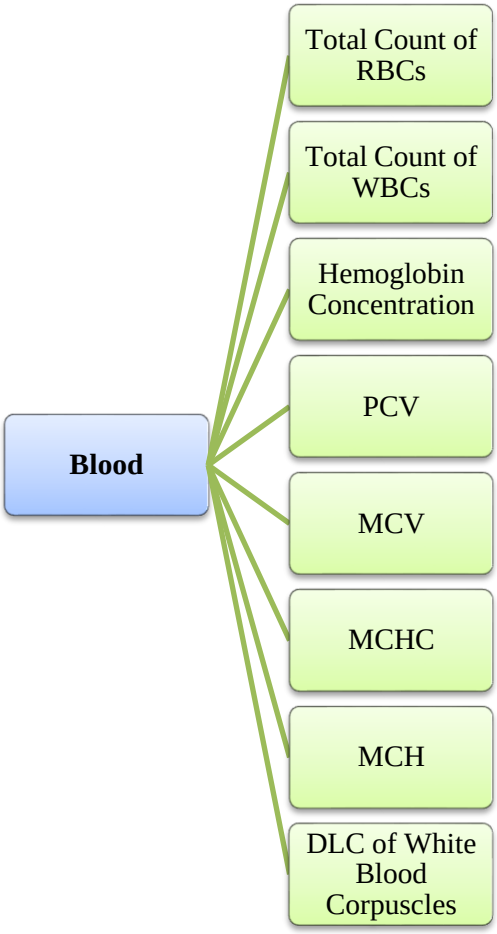
PLAN OF WORK

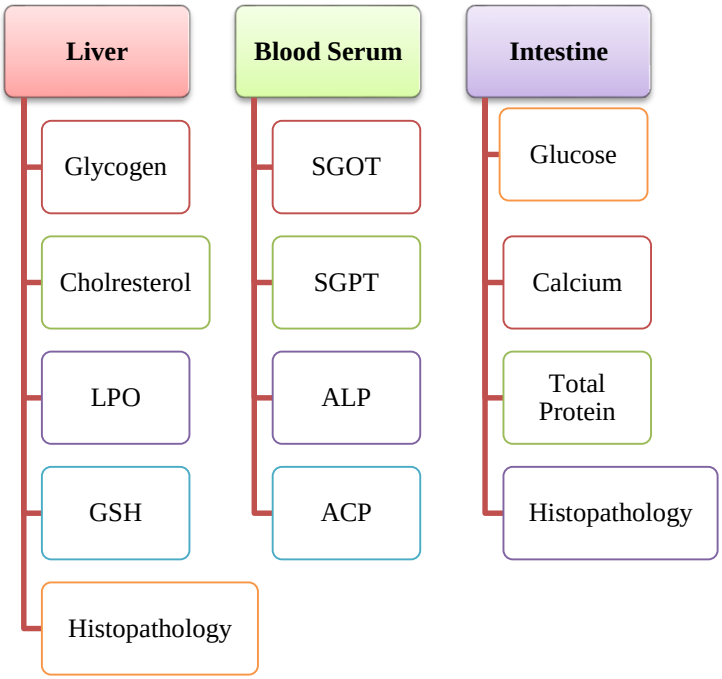

Group of Experimental Animals
Group A (Control)
Group B (Positive Control)
Group C (Experimental)
Experimental I (6 mg/ml dose)
Experimental II (12 mg/ml dose)
Experimental III (24 mg/ml dose)
Group I A (TiO_2)
Group I B (TiO_2 + *Aloe vera*)
Group II A (TiO_2)
Group II B (TiO_2 + *Aloe vera*)
Group III A (TiO_2)
Group III B (TiO_2 + *Aloe vera*)
Autopsy Interval
15 Days
30 Days
45 Days
60 Days

Note:

- 12 mg/ml is a safer amount of nano titanium dioxide

PARAMETERS


Blood
Total Count of RBCs
Total Count of WBCs
Hemoglobin Concentration
PCV
MCV
MCHC
MCH
DLC of White Blood Corpuscles


Liver
Glycogen
Cholresterol
LPO
GSH
Histopathology
Blood Serum
SGOT
SGPT
ALP
ACP
Intestine
Glucose
Calcium
Total Protein
Histopathology

❖ The following parameters which will be studied at autopsy in blood:-

1. Total Count of Red Blood Cells (RBC):

The normal red cell or erythrocyte is elastic, circular; non - nucleated and has the shape of a biconcave disc. The function of the red cell is to transport oxygen through haemoglobin in a functional state. It is expresse in million/cu. mm of blood. The range of red cell diameter (RCD) in health is 6.7 to 7.7 µm. The average thickness of RBC at the periphery is 2.2 µm and at the center is 1 µm.

The dilution of blood 200 times is needed to reduce the number of cells within a given representative fraction that in turn help the counting procedure. Otherwise, the individual cells can hardly distinguish in counting chamber.

The erythrocyte count had performed to determine whether a person has anaemia or polycythaemia. The common causes of high RBC count are high altitude, severe dehydration, shock, immediately after exercise and just after birth. The causes of low RBC count are in mother after delivery of the baby, in starvation, in old age and in all anemias due to various causative factors.

2. Total Count of White Blood Cells (WBC):

The normal white cell or leukocyte is circular and nucleated cells with size larger than RBC (6 to 20 µm in diameter). The various types of white blood cells are neutrophils, basophiles, eosinophils, lymphocytes and monocytes. Leukocytes are need of the body because it show defence against invading organisms like bacteria, viruses and cancer cells. It is express in per cu. mm.

The function of WBC diluting fluid is to destroy RBC and stain the nucleus of WBC to increase the visibility. In WBC diluting fluid, glacial acetic acid causes lysis of red cells and gentian violet or methyl violet slightly stains the nuclei of leukocytes.

When total WBC count is more than 10,000/cu. mm (10×10^9/L) of blood, it is called absolute leukocytosis.

3. Estimation of Hemoglobin Concentration by Cyanmethemoglobin Method:

Hemoglobin is the major constituent of the red cell cytoplasm, accounting for approximately 90% of the dry weight of the mature cell. Four hemin molecules have attached to one large molecule of globin in a conjugated protein of Hemoglobin. Heme has the ability to bind oxygen reversibly and carry it to tissues. It also facilitates the exchange of carbon dioxide between the lungs and tissues. Thus, hemoglobin functions as the primary medium of exchange of oxygen and carbon dioxide.

Blood is dilute in Drabkin's solution containing potassium cyanide and potassium ferricyanide. The latter converts Hemoglobin to methemoglobin that is convert to cyanmethemoglobin (HiCN) by potassium cyanide.

Cell Hemoglobin decreases in hypochromia that causes decrease in MCH level. Hemoglobin is low in anemia, hemorrhages, thalassaemia, hemolytic disorders, and jaundice.

4. Determination of Packed Cell Volume (PCV) by Microhaematocrit Method:

PCV value is the volume of cellular elements per unit volume of whole blood. It is express in percentage.

When any blood sample is centrifuged, then erythrocytes or RBCs separate and settle down. Plasma is seen as a clear liquid. The settled RBCs represent PCV which is referred as haematocrit. True haematocrit is obtained by multiplying observed haematocrit by 0.97 to 0.99, because packing is incomplete to some extent due to trapped plasma.

PCV is use for calculation of absolute values for determination of anemia e.g., MCV and MCHC. PCV increases in polycythemia vera; secondary polycythemia due to chronic cyanotic heart diseases and diminishes in anemia, hemodilution or over hydration. By color of plasma, identify diseases in PCV determination. It is reddish in case of hemolysis and yellowish in case of jaundice.

5. Determination of Mean Corpuscular Volume (MCV):

MCV is the volume of an 'average' RBC. It is express in cubic micron (cu. μ or μ^3).

Levels of MCV are decrease in microcytic anemia sometimes as low as 50 cu. μ and increase in macrocytic anemia sometimes as high as 150 cu. μ.

6. Determination of Mean Corpuscular Hemoglobin Concentration (MCHC):

MCHC means the amount of Hemoglobin present in 100 ml of RBC. This is express as percentage of Hemoglobin in each cell.

Microcytic and macrocytic anemia can assess by the Mean corpuscular hemoglobin concentration. Levels of MCHC are decrease in hypochromic anemia and elevate in macrocytic states and in spherocytosis.

7. Determination of Mean Corpuscular Hemoglobin (MCH):

MCH is the amount of Hemoglobin present in an 'average' RBC. It is expressed in micro micrograms (γγ or gamma gamma) per cell or pictogram per RBC. Normal MCH is indicates the average color (Hemoglobin content) of a single RBC. Variations are express in terms of hypo, hyper or orthochromic (normal) conditions.

Levels of MCH are decrease in hypochromia due to decrease in cell Hemoglobin and increase in macrocytic anemia.

8. Differential Count (DLC)of White Blood Corpuscles:

Differential count (DC) is an index of the relative percentage of different varieties of white blood cells counted on a properly stained blood film from field to field under the microscope.

Methyl alcohol acts as a solvent for Leishman's stain. It fixes the blood film to the slide. It should be acetone free because presence of acetone decolorizes the cells. Methylene blue (basic) stains the nucleus and basophilic granules; and Eosin (acidic) stains the eosinophilic granules.

DLC is significant in estimating values of different cells present in the blood which helps in deducing any disorder of the body, e.g., in any infection, Neutrophil count increases and in allergic condition Eosinophils increase, etc.

❖ The following parameters which will be studied at autopsy in liver:-

1. Glycogen Estimation by Rex Montgomery Method (1957):

Glycogen is branched polysaccharide with 1, 4 - linkage excepting branch point where linkage is 1, 6-carbon.

The tissues are digested in 30% KOH solution. Therefore, the polysaccharide gets dissolved. Now they are precipitated by adding ethanol. They are now treated with conc. H_2SO_4 and 80% phenol, thereby causing dehydration of polysaccharides and finally rearrangement of that molecule takes place to form Polyhydroxy aldehydes which is pink coloured. Thus, the intensity of the colour is directly proportional to the conc. of glycogen.

2. Cholesterol Estimation by Liebermann Burchard Test (1952):

Cholesterol is a steroid derivative and has a basic steucture of a ring. Normal level varies with age, diet and from one location to another.

This method is based on Solowiski reaction in which cholesterol reacts with acetic acid followed by H_2SO_4. A brown coloured complex Disulphonic acid is formed and the intensity of colour is directly proportional to the amount of cholesterol present.

3. Estimation of LPO (Lipid Per Oxidation) by Ohkawa *et al.* (1979):

Lipid per oxidation refers to the oxidative degradation of lipids. It is the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. It most often affects polyunsaturated fatty acids, because they contain multiple double bonds in between which lie

methylene bridges (-CH_2-) that possess especially reactive hydrogen. It is expressed as micron mole per gm of tissue.

4. Estimation of GSH (Glutathione) by Moron *et al.* (1979):

Glutathione (GSH, γ - glutamylcysteinylglycine), the primary non - protein sulfhydryl in aerobic organisms is synthesized in most cells. The ubiquitous tripeptide is formed by the ATP dependent condensation of glutamic acid and cysteine, catalyzed by γ - glutamylcysteinyl synthetase. Glycine is then added by glutathione synthetase to form GSH. In addition to donating an electron during the reduction of hydro peroxides to the respective alcohols (or water in the case of hydrogen peroxide), GSH functions as a co-substrate in the metabolism of xenobiotics catalyzed by glutathione S-transferases. It is also a co-factor for several metabolic enzymes and is involved in intracellular transport, functions as an antioxidant and radioprotectant and facilitates protein folding and degradation. It is expressed as micron mole per mg of tissue.

❖ The following parameters which will be studied at autopsy in blood serum:-

1. Alkaline Phosphatase by Colorimetric Assay Kit:

Alkaline phosphatase (ALP, ALKP, ALPase, Alk Phos) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. The process of removing the phosphate group called dephosphorylation. As the name suggests, alkaline phosphatases are most effective in an alkaline environment. Typical use in the lab for alkaline phosphatases includes removing phosphate monoester to prevent self – ligation (Maxam *et al.,* 1980). Another important use of alkaline phosphatase is as a label for enzyme immunoassays. Humans and most other mammals contain the following alkaline phosphatase isozymes-

ALPI : Intestinal (molecular weight of 150 kDa)

ALPL : Tissue-nonspecific (liver/bone/kidney)

ALPP : Placental (Regan isozyme)

Hypophosphatasia, hypothyroidism, or severe anemia, and chronic myelogenous leukemia are the conditions or diseases may lead to reduced levels of alkaline phosphatase.

2. Acid Phosphatase by Colorimetric Assay Kit:

Acid phosphatase (acid phosphomonoesterase) is a phosphatase, a type of enzyme, used to free attached phosphoryl groups from other molecules during digestion. Acid phosphatase is stored in lysosomes and functions when these fuse with endosomes, which are acidify while they function; therefore, it has an acid pH optimum (Henneberry *et al.*, 1979).

Different forms of acid phosphatase is find in different organs, and their serum levels is use to evaluate the success of the surgical treatment of prostate cancer (Henneberry *et al.,* 1979).

3. Serum Glutamic - Pyruvic Transaminase (SGPT) by IFCC (International Federation of Clinical Chemistry) Method:

SGPT is an enzyme that is normally present in liver and heart cells. SGPT is release into blood when the liver or heart is damage. It also called alanine aminotransferase (ALT) or alanine aminotransferase (ALAT). It catalyzes the two parts of the alanine cycle. ALT catalyzes the transfer of an amino group from L - alanine to α - ketoglutarate, the products of this reversible transamination reaction being pyruvate and L - glutamate.

ALT is commonly measure clinically as a part of a diagnostic evaluation of hepato - cellular injury, to determine liver health. The blood SGPT levels are thus elevated with liver damage (for example, from viral hepatitis) or with an insult to the heart (for example, from a heart attack). Some medications can also raise SGPT levels.

4. Serum Glutamic Oxaloacetic Transaminase (SGOT) by IFCC (International Federation of Clinical Chemistry) Method:

SGOT is an enzyme that is normally present in liver and heart cells. SGOT is release into blood when the liver or heart is damage. SGOT also called aspartate aminotransferase (AST).

The blood SGOT levels are thus elevated with liver damage (for example, from viral hepatitis) or with an insult to the heart (for example, from a heart attack). Some medications can also raise SGOT levels.

❖ The following parameters which have been studied at autopsy in intestine:-

1. Total Protein by Lowry's Method:

Total protein is a biochemical test for measuring the total amount of protein in serum and intestine. Protein made up of albumin and globulin. The measurement usually performed on automated analyzers.

2. Intestinal Glucose:

Glucose is a sugar with the molecular formula $C_6H_{12}O_6$. It also called dextrose or grape sugar. With six carbon atoms, it is class as a hexose, a sub - category of monosaccharides. The D - isomer (D - glucose) occurs widely in nature, but the L - isomer (L - glucose) does not. Glucose is stored as a polymer, in plants as starch and in animals as glycogen.

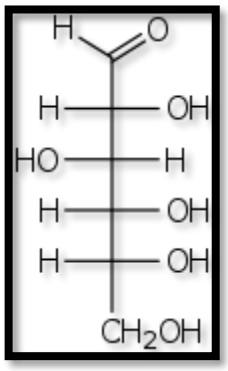


Figure 5: D-glucose in fisher projection

3. Intestinal Calcium:

Calcium is a chemical element with symbol Ca and atomic number 20. Calcium is a soft gray alkaline earth metal, fifth - most - abundant element by mass in the Earth's crust. Calcium is essential for living organisms, in particular in cell physiology, where movement of the calcium ion into and out of the cytoplasm functions as a signal for many cellular processes.

❖ The following organs which have been studied at autopsy for histopathology:-

1. Liver
2. Intestine

Principle:

Histopathology (compound of three Greek words: *histos* "tissue", *pathos* "suffering", and *logia* "study of") refers to the microscopic examination of tissue in order to study the manifestations of disease. Specifically, in clinical medicine, histopathology refers to the examination of a biopsy or surgical specimen by a pathologist, after the specimen has processed and histological sections have placed onto glass slides.

The tissue is removing from the body, and then placed in a fixative that stabilizes the tissues to prevent decay. The most common fixative is formalin (10% formaldehyde in water).

SIGNIFICANCE OF THE STUDY

The study will help in identifying the toxic nature (if any) of the most prevalently used nano substance titanium dioxide in foodstuffs and its implications and further, in curing and nullifying these effects with the commonly used medicinal plant *Aloe vera.*

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